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KEY TO THE SUCCESSFUL RNA INTERFERENCE (RNAI) MEDIATED MANAGEMENT OF AGRICULTURAL PESTS

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ABSTRACT

The threat posed by insect pests to agricultural production is the major adversity that led to food grains scarcity. Blocking of gene functions using RNA interference (RNAi) is one of the current alternates for controlling of agricultural pests. Of late, research on RNAi mediated controlling of agricultural pests has made significant growth. However, accumulated studies revealed that implementation of RNAi for management of agricultural pests is still challenging and not matching with its envisioned potential. Therefore, understanding the factors influencing the RNAi efficiency consequently aid in designing an effective RNAi strategy. This review provides insights into the factors governing the RNAi. Overall, the RNAi success is governed by the sensitivity of target insect, selection of target gene, mode of delivery of double-stranded RNA (dsRNA), the length, concentration and persistence of dsRNA in the target pests.

KEYWORDS: RNAi, Insects, dsRNA, dsRNA Delivery, Core RNAi Pathway Genes

INTRODUCTION

The threat posed to crop production by insect pests is one the key factors that could threaten to destabilize global food security. The food grain production saw a decline during last decade and it has become exacerbated in the recent years that evident in price escalation of daily use food commodities. Agricultural pests are one of the major threats in the declining of global agricultural food grain production. Therefore, controlling of insect pests is one of the major tasks in order to feed the human population.

RNAi is a post-transcriptional gene silencing mechanism triggered by the introduction of double-stranded RNA (dsRNA) or by endogenous dsRNA that led to the sequence-specific degradation of target mRNA thus impeding the target gene functions. From the discovery of RNAi in the nematode, *Caenorhabditis elegans*, its applications are ever-increasing in various fields including agriculture and medicine (Fire et al., 1998; Sharath Chandra et al., 2012). RNAi is one of the major tools in reverse genetics as it is been used in the functional characterization of genes and it also becoming a new arsenal in the management of agricultural pests.

Mechanism of RNAi

RNAi mechanism is elicited by dsRNA which is either exogenous and/or endogenous in origin and entered dsRNA gets diced into 20-25 bp fragments called small interfering RNA (siRNA) by the action of RNase III nuclease called Dicer,

then siRNAs are incorporated into the multi-enzyme complex know as RNA induced silencing complex (RISC). The incorporated siRNA strand guides the RISC to the cognate mRNA, Consequently, RISC complex binds and degrades the cognate mRNA using its catalytic Argonaute (Ago) proteins (Filipowicz, 2005).

Factors Governing the RNAi Efficiency

RNAi efficiency is governed by a number of factors that include target insect, selection of gene, mode of dsRNA delivery, the length of dsRNA, concentration and persistence of dsRNA and core components of RNAi pathway.

Target Insect

RNAi experiments were extensively conducted in Orthoptera, Blattodea, Hemiptera, Coleoptera, Neuroptera, Hymenoptera, Lepidoptera and Diptera order insects (Table 1). However, very few studies or no studies are performed in Archaeognatha, Thysanura, Ephemeroptera and Odonata order insects (Belles, 2010). RNAi efficiency appears to vary significantly among the insect orders due to the disparities in the intrinsic nature and properties of insect species. RNAi studies on Coleoptera order insects revealed that these insects, especially red flour beetle (*Tribolium castaneum*, western corn rootworm (*Diabrotica virgifera virgifera*) and Colorado potato beetle (*Leptinotarsa decemlineata*) were very sensitive to RNAi. While Diptera and Lepidoptera order insects were found to be less sensitive to RNAi. In general, the less developed insect species (*T. castaneum*) were more sensitive to RNAi compared to sophisticatedly developed insects (Lepidoptera). The well-known poor responsive insect is *Drosophila melanogaster* (Katoch and Thakur, 2013).

Mode of dsRNA Delivery

Feasibility and efficiency of RNAi in controlling insect pests is strongly influenced by mode of dsRNA delivery. Delivery of dsRNA to target insects is mainly achieved through microinjection or spraying or through diet. Microinjection is commonly used in the laboratory experiment to deliver dsRNA to test insect and this method was efficient and researchers can have control over the amount of dsRNA delivered to insect (Jing and Zhao-Jun, 2014). Despite extensive use of this method in laboratory experiments, it is not feasible for field-level insect pest control. Moreover, microinjection is not possible if the test insect is very small (Kumar and Puttaraju, 2012). On the other hand, delivery of dsRNA through the diet via oral route mimics the natural way of dsRNA uptake and also facilitates high-throughput RNAi screening. Diet-mediated delivery of dsRNA is a simple approach achieved either by feeding the insect on dsRNA incorporated diet or on dsRNA expressing genetically modified plants. Feeding of larvae on a diet containing dsRNA is one of the simplest approaches in silencing of the target genes and facilitates high-throughput screening of various target genes. However, the major limitation of this method is difficult to assess the amount dsRNA fed by larvae. Moreover, the stability of dsRNA in the diet is greatly influenced by the composition of the diet. Nevertheless, feeding of larvae on dsRNA expressing RNase III mutant (HT115) strain of *Escherichia coli* bacteria facilitates intact dsRNA delivery into the test insect (Timmons et al. 2001). Feeding of *H. armigera* larvae on bacteria expressing ultraspiracle dsRNA resulted in higher larval mortality as compared to larvae fed on naked dsRNA (Yang and Han 2014). Therefore, this approach enhances the RNAi effectiveness due to the protection of expressed dsRNA afforded by cell wall of *E. coli*. Although, some studies have revealed that feasibility of this method depends on the optimization of feeding protocol. Recently, delivery of dsRNA to insects also been achieved either by topical application or spraying. The

topical application/spraying of dsRNA is best achieved through nanoparticle-mediated dsRNA delivery. Nanoparticle-mediated dsRNA delivery is an effective method for delivering dsRNA into insects and this also protect the dsRNA and aid in slow release of dsRNA in the insect body. In this regard, chitosan coated dsRNA was successfully delivered to mosquito larvae (Zhang et al., 2010). In another study, delivery of chitosan nanoparticle coated Acetylcholinesterase (AChE2) siRNAs caused larval mortality in the case of diamondback moth, *Plutella xylostella* (Gong et al 2013). However, currently this method of dsRNA delivery to test insect is still in the infancy stage. On the other hand, engineering of plants to produce specific dsRNA is one of the alternate and facilitates the persistent silencing of target genes. To impart resistance to plants against bollworm, *H. armigera*, authors have engineered *Arabidopsis thaliana* plants to produce dsRNA of a CYP6AE14 isoform of cytochrome P450. Feeding of larvae on these plants manifested in larval growth inhibition and eventually caused larval mortality (Mao et al., 2007). From this point onwards so much progress has been made in enhancing dsRNA expression in the transgenic plants. In this regard, high throughput dsRNA expression systems have been developed. In a previous study, we have developed a modular vector for assembling hairpin construct of sense and antisense fragment of the gene in single step (Manamohan et al., 2013). Recently, engineering of chloroplasts DNA (transplastomic) with hairpin construct of sense and antisense of insect genes for dsRNA expression has resulted in higher expression of dsRNA in chloroplast transformants as compared to dsRNA expressed in nuclear transformants (Jin et al., 2015; Zhang et al., 2015). The higher expression of dsRNA in chloroplast was attributed due to the polyploidy nature of plastid genetic system and this enables the production of up to 10,000 copies of gene of interest per cell (Ruiz et al. 2011). Compared to other methods transgenic plants constantly produce targeted dsRNA that ensures continuous availability of dsRNA to test insects.

Selection of Gene

The selection of gene greatly influences the success rate of RNAi in pest control. The gene for RNAi should be selected based on its biological role in the insect growth and development. RNAi mediated screening of 290 genes of western corn rootworm (*Diabrotica virgifera virgifera*) revealed that only 125 genes were able to cause larval mortality (Baum et al., 2007). In this study, the most effective target genes were vacuolar ATPase (v-ATPases) subunits A and D and ribosomal protein. Further authors have developed corn transgenic plants to express dsRNA of v-ATPases subunit A. Feeding of larvae on these plants caused larval death. In a previous study, only three genes were showed higher silencing out of five tested genes of *H. armigera* (Asokan et al., 2014). The compilation of RNAi experiments in Lepidoptera order insects demonstrated that out of 130 genes only 38% (49) genes were silenced more than 50% whereas 14 % (18) genes were showed lesser silencing while 48% (62) genes did not silence at all (Terenius et al., 2011). Overall, genes involved in immune response have shown higher silencing while the genes involved in protein-protein interactions shown lower silencing. Sometimes, knockdown of very crucial genes especially those involved in regulation of hormone biosynthesis subsequently elicits the feed-back mechanism in order to impede the silencing effect. This feedback mechanism counteracts the silencing effect either by over-expressing the target gene or up-stream genes in order to produce more precursors. In a previous study, silencing of juvenile hormone acid methyltransferase, (*jhamt*) which is involved in regulation of juvenile hormone biosynthesis resulted in over-expression of upstream gene, farnesyl diphosphate synthase (*fpps*) which compensated the silencing effect on *H. armigera* (Asokan et al., 2013). Similarly, continuous feeding of dsRNA to the tephritid fruit fly (*Bactrocera dorsalis)* showed over-expression of the target gene (fatty acid elongase-*noa*) (Li et al., 2011). Therefore, RNAi efficiency greatly hinges on the selection of gene and

careful considerations needs to be followed while selecting gene as a target for RNAi mediated controlling of insect pests.

Concentration of dsRNA

In insects, RNAi could be elicited by delivering dsRNA, though the amount/concentration of dsRNA required to elicit RNAi in different insect species is highly variable. RNAi studies in insects were performed by delivering 1 µg to 100 µg concentration of dsRNA. In some insects, Cecropia Moth (*Hyalophora cecropia*), Chinese (Oak) tussah moth (*Antheraea pernyi*) and tobacco hornworm (*Manduca sexta*) even 10 ng concentration of dsRNA per mg of insect biomass was sufficient to elicit high levels of target gene silencing (Bettencourt et al., 2002; Hirai et al., 2004; Terenius et al., 2007). Similarly, silencing of target genes in the Coleoptera order beetles could be achieved with small quantities of dsRNA (Palli, 2014). Contrastingly, very high concentration (100 µg) of dsRNA per mg of insect biomass was required to silence target gene of tussah moths (*Antheraea mylitta*) (Gandhe et al., 2007). Despite these variations, delivery of dsRNA through microinjection requires less concentration of dsRNA as compared to delivering dsRNA through diet which requires higher concentrations of dsRNA. This could be due to the dsRNA delivered through diet gets diluted or degraded. In this regard, of late studies revealed that insect midgut juices possess high quantities of dsRNA degrading enzymes (dsRNase) as compared to hemolymph (Christiaens et al., 2014). In the case of microinjection, dsRNA directly delivered into hemolymph and dsRNA immediately transverses to different organs of the test insect and RNAi being elicited immediately. Therefore, time lag after dsRNA delivery to RNAi elicitation was lesser in the case of microinjection as compared to administration of dsRNA through feeding. Further, in some insects, diet-delivered dsRNA was inefficient in silencing of target genes which expressed away from the midgut.

In insects, dsRNA effect appears to be transient in nature, i.e. the administered dsRNA effect on test insect persisted for a short period of time (Liu et al., 2010). This might be due to the absence of RNA dependent RNA polymerase (RdRP) which is essential for RNAi signal amplification, i.e. production of secondary siRNAs from primary siRNAs cleaved mRNAs, the cleaved mRNA act as a substrate for RdRP which produces secondary siRNA and they again cleave the target mRNA and this process gets repeated. In the nematode, *Caenorhabditis elegans* RNAi effect inherits to next generations due to the presence of RdRP (Grishok et al., 2000). Nevertheless, there was no report of RdRP from insects, therefore, influencing the persistence of dsRNA and its effects on target gene silencing. Thus, the extent of target gene silencing appears to be highly dependent on the concentration of dsRNA and it is likely that there is no uniform dsRNA concentration that work across the insect species.

Length of dsRNA

The efficacy of RNAi hinges on the length of dsRNA sequence. In this regard, the length of dsRNA is one of the critical parameters that determines dsRNA uptake and silencing efficiency (Huvenne and Smagghe, 2010; Garbutt and Reynolds, 2012). In this regard, long dsRNA (300-520 bp) was found to be more effective than siRNA (21 bp) and shorter (31 bp) dsRNA in silencing of target genes of *T. castaneum* (Miller et al., 2012; Wang et al., 2013). This was probably due to the inefficient uptake of siRNA as evident in *D. melanogaster* cells (Saleh et al., 2009). Generally, long dsRNA act as a substrate for Dicer thus evokes an efficient RNAi response and lasts for longer duration even at low concentrations of dsRNA treatment (Kim et al., 2005). Since, RNAi has evolved as a natural defense mechanism against the invading viruses, the host system

distinguishes the self and non-self (viral) RNAs based on the length of dsRNA. In this regard, invading viruses generates long dsRNAs (>1-2 kb) that exceeds the average length of self RNAs (<0.5 kb), hence, the length dependent activation of antiviral signaling occurs, that consequently activates the immune system (Peisley et al., 2012).

Therefore, the ideal length of dsRNA molecule to trigger efficient RNAi varies greatly among the insect species, though, great success in insect RNAi experiments were obtained with 300-520 bp length of dsRNA (Huvenne and Smagghe, 2010). However, recent investigations revealed that the long dsRNAs had unintended off-target effects such as cross-suppression of closely related genes within the organism or in related species (Araujo et al., 2006). Efforts in minimizing RNAi off-target effects has seen the development of software's such as siDirect (Naito et al. 2004), dsCheck (Naito et al. 2005) and off-target finder (http://146.118.96.106/rnai/). Further, the selected sequences should be subjected to global blast analysis in order to minimize off-target effects.

Core RNAi machinery

Despite the above factors, the difference in the insect sensitivities to RNAi appears to be due to the varied response of core RNAi pathway genes. Generally, the efficiency of RNAi is governed by RNAi pathway genes such as Dicer-1, Dicer-2, argonaute-1 (Ago-1), and Ago-2 (Garbutt and Reynolds, 2012; Li et al., 2015). In this regard, Dicer-1 and Dicer-2 are involved in processing of long dsRNAs into miRNA and siRNAs, respectively while Ago-1 and Ago-2 are involved in cleaving of the target mRNA (Filipowicz, 2005). In insects, very little is known about the regulation of RNAi pathway genes in response to exogenously supplied dsRNA. The assessment of RNAi pathway genes (Dicer-2 and Ago-2) response to exogenously delivered dsRNA revealed that within 6 hours after administering dsRNA, these genes expression elicited and persisted for only short period of time (up to 24 hours) then they fell back to basal levels (Garbutt and Reynolds, 2012). Additionally, the second time administration of dsRNA into *M. sexta* manifested in once again elevation of Dicer-2 and Ago-2 expression. In our previous study, single time administration of dsRNA caused target gene silencing only for one day and next day the silencing levels were drastically dropped while multiple applications of dsRNA led to higher and persistent silencing of target genes of *H. armigera* (Asokan et al., 2013). Notably, over-expression of Ago-2 in silkworm (*Bombyx mori*) resulted in enhanced RNAi effect (Li et al., 2015). Therefore, there seems to be a direct correlation between the expression levels of Dicer-2 and Ago-2 and extent of target gene silencing. Therefore, to maintain the higher levels of Dicer-2 and Ago-2 and to prolong the dsRNA effect, continuous administration of dsRNA perhaps prove to be useful for enhancing RNAi effect on agricultural pests.

CONCLUSIONS AND FUTURE REMARKS

The proof-of-principle for RNAi based controlling of agricultural pests has been demonstrated. However, RNAi technology is still in infancy stage in the management of agricultural pests due to cryptic understanding of the factors governing the RNAi efficiency. Nevertheless, RNAi is envisioned to be a potential tool in the management of agricultural pests due to its specificity and further exploration of critical factors governing RNAi efficiency eventually aid in the enhancement of RNAi potentiality. The success rate of RNAi could be enhanced by selecting a biologically crucial target gene and refining dsRNA molecule and its delivery method. The efforts are needed to reinforce the RNAi effectiveness on agricultural pests by silencing multiple genes using combinatorial dsRNA molecules. Further, the development of transplastomic (transgenic plants harboring gene of interest in the chloroplast DNA) in order to express high amount of dsRNA opens the new avenue for

effective controlling of agricultural pests and this also minimizes the cross contamination of wild type plants.

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REFERENCES

1. Amdam, G.V., Simoes, Z.L., Guidugli, K.R., Norberg, K. & Omholt, S.W. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnology*, 3, 1.
2. Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J. & Pereira, M.H. (2006). RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. *Insect Biochemistry Molecular Biology*, 36, 683-693.
3. Aronstein, K., Pankiw, T. & Saldivar, E. (2006). SID-1 is implicated in systemic gene silencing in the honey bee. *Journal of Apicultural Research*, 45, 20–24.
4. Asokan, R., Sharath Chandra, G., Manamohan, M. & Kumar, N.K. (2013). Effect of diet delivered various concentrations of double-stranded RNA in silencing a midgut and a non-midgut gene of *Helicoverpa armigera*. *Bulletin of Entomological Research*, 103, 555–563.
5. Asokan, R., Sharath Chandra, G., Manamohan, M., Kumar, N.K. & Sita, T. (2014). Response of various target genes to diet-delivered dsRNA mediated RNA interference in the cotton bollworm, *Helicoverpa armigera*. *Journal of Pest Science*, 87, 163-172.
6. Attardo, G.M., Higgs, S., Klingler, K.A., Vanlandingham, D.L. & Raikhel, A.S. (2003). RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences* U.S.A, 100, 13374-13379.
7. Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O. et al. (2007). Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, 25, 1322-1326.
8. Belles, X. (2010). Beyond Drosophila: RNAi in vivo and functional genomics in insects. *Annual Review of Entomology*, 55, 111–128.
9. Bettencourt, R., Terenius, O. & Faye, I. (2002). Hemolin gene silencing by ds-RNA injected into *Cecropia* pupae is lethal to next generation embryos. *Insect Molecular Biology*, 11, 267–271.
10. Bucher, G., Scholten, J. & Klingler, M. (2002). Parental RNAi in *Tribolium* (Coleoptera). *Current Biology*, 12, R85-R86.
11. Christiaens, O., Swevers, L. & Smagghe, G. (2014). DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides*, 53, 307-314.
12. Ciudad, L., Piulachs, M.D. & Belles, X. (2006). Systemic RNAi of the cockroach vitellogenin receptor results in a

phenotype similar to that of the *Drosophila* yolkless mutant. *FEBS Journal*, 273, 325–35.

13. Dong, Y. & Friedrich, M. (2005). Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. *BMC Biotechnology*, 5, 25.
14. Filipowicz, W. (2005). RNAi: the nuts and bolts of the RISC machine. *Cell*, 122, 17–20.
15. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. & Mello C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806–811.
16. Gandhe, A.S., John, S.H. & Nagaraju, J. (2007). Noduler, a novel immune up-regulated protein mediates nodulation response in insects. *Journal of Immunology*, 179, 6943–6951.
17. Garbutt, J.S., Reynolds, S.E. (2012). Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference. *Insect Biochemistry and Molecular Biology*, 42 (9), 621-628.
18. Gong, L., Chen, Y., Hu, Z., Hu, M. (2013). Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. *PLoS ONE*, 8(5):e62990.
19. Grishok, A., Tabara, H. & Mello, C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science*, 287, 2494–2497.
20. Hirai, M., Terenius, O. & Faye, I. (2004). Baculovirus and dsRNA induce hemolin, but no antibacterial activity, in *Antheraea pernyi*. *Insect Molecular Biology*, 13, 399–405.
21. Huvenne, H. & Smagghe G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of Insect Physiology*, 56, 227–235.
22. Jaubert-Possamai, S., Le Trionnaire, G., Bonhomme, J., Christophides, G.K., Rispe, C., & Tagu, D. (2007). Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. *BMC Biotechnolology*, 7, 63.
23. Jin, S., Singh, N.D., Li, L., Zhang, X. & Daniell, H. (2015). Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts Helicoverpa armigera larval development and pupation. *Plant Biotechnology Journal*, 13, 435–446.
24. Jing, Y. & Zhao-jun, H. (2014). Efficiency of Different Methods for dsRNA Delivery in Cotton bollworm (*Helicoverpa armigera*). *Journal of Integrative Agriculture*, 13(1), 115-123.
25. Katoch, R. & Thakur, N. (2013). Advances in RNA interference technology and its impact on nutritional improvement, disease and insect control in plants. *Applied Biochemistry and Biotechnology*, 169(5), 1579-1605.
26. Kim, D.H., Behlke, M.A., Rose, S.D., Chang, M.S., Choi, S. & Rossi, J.J. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nature Biotechnology*, 23, 222–226.
27. Kumar, M., Gupta, G.P. & Rajam, M.V. (2009). Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. *Journal of Insect Physiology*, 55(3), 273–278.

28. Kumar, S.S. & Puttaraju, H.P. (2012). Improvised microinjection technique for mosquito vectors. *Indian Journal of Medical Research*, 136, 971-978.

29. Li, X., Zhang, M. & Zhang, H. (2011). RNA Interference of four genes in Adult *Bactrocera dorsalis* by feeding their dsRNAs. *PLoS ONE*, 6, e17788.

30. Li, Z., Zeng, B., Ling, L., Xu, J., You, L., Aslam, A.F., Tan, A. & Huang, Y. (2015). Enhancement of Larval RNAi Efficiency by Over-expressing Argonaute2 in *Bombyx mori*. *International Journal of Biological Sciences*, 11(2), 176-185.

31. Liu S, Ding Z, Zhang C, Yang B, Liu Z (2010) Gene knockdown by introthoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*. *Insect Biochemistry Molecular Biology*, 40, 666–671.

32. Lynch, J.A. & Desplan, C. (2006). A method for parental RNA interference in the wasp *Nasonia vitripennis*. *Nature Protocols*, 1, 486-494.

33. Manamohan, M., Sharath Chandra, G., Asokan, R., Deepa, H., Prakash, M.N, & Krishna Kumar, N.K. (2013). One-step DNA fragment assembly for expressing Intron containing hairpin RNA in plants for gene silencing. *Analytical Biochemistry*, 433, 189–191.

34. Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P. & Chen, X.Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology*, 25, 1307–1313.

35. Miller, S.C., Miyata, K., Brown, S.J. & Tomoyasu, Y. (2012). Dissecting Systemic RNA Interference in the Red Flour Beetle *Tribolium castaneum*: Parameters Affecting the Efficiency of RNAi. *PLoS ONE*, 7(10), e47431.

36. Misquitta, L. & Paterson, B.M. (1999). Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proceedings of the National Academy of Sciences U.S.A*, 96, 1451-1456.

37. Miyawaki, K., Mito, T., Sarashina, I., Zhang, H., Shinmyo, Y., Ohuchi, H. & Noji, S. (2004). Involvement of Wingless/Armadillo signaling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed by RNAi analysis. *Mechanisms of Development*, 121, 119-130.

38. Naito, Y., Yamada, T., Ui-Tei, K., Morishita, S., & Saigo, K. (2004). siDirect: Highly effective, target-specific siRNA design software for mammalian RNA interference. *Nucleic Acids Research*, 32: W124–W129.

39. Naito, Y., Yamuda, T., Mastumiya, T., Kumiko, U.T., Saigo, K. & Morishita, S. (2005). dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. *Nucleic Acids Research*, 33, W589-W591.

40. Palli, S.R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide, *Current Opinion in Insect Science*, 6, 1-8.

41. Peisley, A., Jo, M.H., Lin, C., Wu, B., Orme-Johnson, M., Walz, T., Hohng, S. & Hur, S. (2012). Kinetic mechanism for viral dsRNA length discrimination by MDA5 filaments. *Proceedings of the National Academy of Sciences U.S.A*, 109, E3340–E3349.

42. Ruiz, O.N., Alvarez, D., Torres, C., Roman, L. & Daniell, H. (2011). Metallothionein expression in chloroplasts enhances mercury accumulation and phytoremediation capability. *Plant Biotechnology Journal*, 9, 609–617.

43. Saleh, M.C., Tassetto, M., van Rij, R.P., Goic, B., Gausson, V., Berry, B., Jacquier, C., Antoniewski, C. & Andino, R. (2009). Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature*, 458, 346–351.

44. Shakesby, A.J., Wallace, I.S., Isaacs, H.V., Pritchard, J., Roberts, D.M. & Douglas, A.E. (2009). A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology*, 39, 1–10.

45. Sharath Chandra, G., Asokan, R. & Manamohan, M. (2012). Modified competing polymerase chain reaction primer for single tube quantitative PCR. *Analytical Biochemistry*, 427(2), 175-177.

46. Terenius, O., Bettencourt, R., Lee, S.Y., Li, W., Soderhall, K. & Faye, I. (2007). RNA interference of hemolin causes depletion of phenoloxidase activity in *Hyalophora cecropia. Developmental and Comparative Immunology*, 31, 571–575.

47. Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H. et al. (2011). RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology*, 57, 231–245.

48. Timmons, L., Court, D.L. & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene, 263, 103–112.

49. Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P. & Newcomb, R.D. (2006). RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Molecular Biology*, 15, 383–391.

50. Walshe, D.P., Lehane, S.M., Lehane, M.J. & Haines, L.R. (2009). Prolonged gene knockdown in the tsetse fly *Glossina* by feeding double stranded RNA. *Insect Molecular Biology*, 18, 11–19.

51. Wang, J., Wu, M., Wang, B. & Han, Z. (2013). Comparison of the RNA interference effects triggered by dsRNA and siRNA in *Tribolium castaneum*. Pest Management Science 69(7), 781-786.

52. Xiong, Y., Zeng, H., Zhang, Y., Xu, D. & Qiu, D. (2013). Silencing the HaHR3 Gene by Transgenic Plant-mediated RNAi to Disrupt *Helicoverpa armigera* Development. *International Journal of Biological Sciences*, 9(4), 370–381.

53. Yang, J. & Han, Z. (2014). Efficiency of Different Methods for dsRNA Delivery in Cotton Bollworm (*Helicoverpa armigera*). *Journal of Integrative Agriculture*, 13(1), 115–123.

54. Zhang, J., Khan, S.A., Hasse, C., Ruf, S., Heckel, D.G. & Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science*, 347, 991–994.

55. Zhang, X., Zhang, J. & Zhu, K.Y. (2010). Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). *Insect Molecular Biology*, 19:683–693.

56. Zhao, Y.Y., Yang, G., Wang-Pruski, G. & You, M.S. (2008). *Phyllotreta striolata* (Coleoptera: Chrysomelidae): arginine kinase cloning and RNAi-based pest control. *European Journal of Entomology* 105, 815–822.

57. Zhou, X., Oi, F.M. & Scharf, M.E. (2006). Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. *Proceedings of the National Academy of Sciences U.S.A*, 103, 4499-4504.

58. Zhou, X., Wheeler, M.M., Oi, F.M. & Scharf, M.E. (2008). RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochem. Molecular Biology*, 38, 805-815.

Table 1: Some RNAi Studies in different Order Insects

Insect Order	Target insect	Target Gene	Reference
Coleoptera	*Diabrotica virgifera virgifera*	Vacuolar ATPase subunit A and E	Baum et al. (2007)
		α-Tubulin	
	Diabrotica undecimpuctata howardii	Vacuolar ATPase subunit A and E	
		α-tubulin	
	Leptinotarsa decemlineata	Vacuolar ATPase subunit A and E	
	Phyllotreta striolata	Arginine kinase	Zhao et al. (2008)
	Tribolium castaneum	Distalless, maxillopedia,	Bucher et al. (2002)
Diptera	*Glossina morsitans morsitans*	Midgut protein TsetseEP	Walshe et al. (2009)
		Transferrin	
	Drosophila	Nautilus gene	Misquitta and Paterson (1999)
	Aedes aegypti	GATA factor	Attardo et al. (2003)
Hemiptera	*Acyrthosiphon pisum*	Water specific aquaporin	Shakesby et al. (2009)
	Rhodnius prolixus	Nitroporin 2	Araujo et al. (2006)
	Acyrthosiphon pisum	Calreticulin	Jaubert-Possamai et al. (2007)
		Cathepsin-L	
Hymenoptera	*Apis mellifera*	Toll-related receptor 18W	Aronstein et al. (2006)
		Vitellogenin	Amdam et al. (2003)
	Nasonia vitripennis	Nanos	Lynch and Desplan (2006)
Isoptera	*Reticulitermes flavipes*	Cellulose enzyme	Zhou et al. (2008)
		Caste regulatory hexamerin storage protein	
	Reticulitermes termites	Hexamerin	Zhou et al. (2006)
Lepidoptera	*Epiphyas postvittana*	Carboxylasterase	Turner et al. (2006)
		Pheromone binding protein	
	Helicoverpa armigera	Cytochrome P450 (CYP6AE14)	Mao et al. (2007)
		Gluthatione-S-transferase	
		Acetylcholinesterase	Kumar et al. (2009)
		Trypsin	Asokan et al. (2014)
		Chymotrypsin	
		Juvenile hormone acid methyltransferase	
		Cytochrome P450	
		HaHR3	Xiong et al. (2013)
Blattodea	*Cockroach*	Vitellogenin	Ciudad et al. (2006)
Orthoptera	*Gryllus bimaculatus*	G. bimaculatus wingless (Gbwg)	Miyawaki et al. (2004)
	Schistocerca americana	Vermilion	Dong and Friedrich (2005)